Effect of lipid extraction on analyses of stable carbon and stable nitrogen isotopes in coastal organisms of the Aleutian archipelago

M.A. Ricca, A.K. Miles, R.G. Anthony, X. Deng, and S.S.O. Hung

Abstract: We tested whether extracting lipids reduced confounding variation in $\delta^{13}C$ and $\delta^{15}N$ values by analyzing paired lipid-extracted (LE) and non-lipid-extracted (NLE) samples of bald eagle (*Haliaeetus leucocephalus* (L., 1766)) whole eggs, muscle tissue from nine seabird and one terrestrial bird species, muscle tissue from four marine fish species, and blue mussels (*Mytilus edulis* L., 1758) collected from the Aleutian archipelago, Alaska. Lipid extraction significantly increased $\delta^{13}C$ by an average of 2.0‰ in whole eggs, 0.8‰ in avian muscle, 0.2‰ in fish muscle, and 0.6‰ in blue mussels. Lower $\delta^{13}C$ values in NLE samples covaried positively with lipid content across all sample types. Lower $\delta^{13}C$ values in NLE samples were not correlated with lipid content within bald eagle eggs and blue mussels, but covaried positively with percent lipid in avian and fish muscles. Neither lipid extraction nor percent lipid significantly changed $\delta^{15}N$ values for any sample type. Lower $\delta^{13}C$ values in most NLE avian and fish muscle tissues should not confound interpretation of pelagic versus nearshore sources of primary production, but lipid extraction may be necessary when highly precise estimates of $\delta^{13}C$ are needed. Lipid extraction may not be necessary when only $\delta^{15}N$ is of interest.

Résumé : Nous vérifions si l'extraction des lipides réduit la variation confondante dans les valeurs de $\delta^{13}C$ et de $\delta^{15}N$ en analysant des échantillons appariés avec (LE) et sans (NLE) extraction de lipides d'oeufs entiers de pygargues à tête blanche (*Haliaeetus leucocephalus* (L., 1766)), de tissus musculaires de neuf espèces d'oiseaux marins et d'une espèce d'oiseau terrestre, de tissus musculaires de quatre espèces de poissons marins ainsi que de moules bleues (*Mytilus edulis* L., 1758) provenant de l'archipel des Aléoutiennes, Alaska. L'extraction des lipides augmente significativement la valeur de $\delta^{13}C$ d'en moyenne 2,0 ‰ dans les oeufs entiers, de 0,8 ‰ dans le muscle aviaire, de 0,2 ‰ dans le muscle de poissons et de 0,6 ‰ dans les moules bleues. Il y a une corrélation positive entre les valeurs réduites de $\delta^{13}C$ dans les échantillons NLE et le contenu lipidique dans tous les types d'échantillons. Les valeurs réduites de $\delta^{13}C$ ne montrent pas de corrélation avec le contenu lipidique au sein des échantillons NLE des oeufs de pygargues ni de moules bleues; il y a cependant une corrélation positive avec le pourcentage de lipides dans les muscles d'oiseaux et de poissons. Dans tous les échantillons, ni l'éxtraction de lipides, ni le pourcentage de lipides ne changent significativement les valeurs de $\delta^{15}N$. Les valeurs réduites de $\delta^{13}C$ dans la plupart des échantillons NLE de muscles d'oiseaux et de poissons ne devraient pas fausser la distinction entre les sources pélagiques et côtières de production primaire; cependant, l'extraction des lipides peut être nécessaire lorsqu'on a besoin d'estimations très précises de $\delta^{13}C$. L'extraction des lipides peut ne pas être nécessaire lorsqu'on s'intéresse seulement à $\delta^{15}N$.

[Traduit par la Rédaction]

Introduction

Sources of primary production and trophic status in food webs can be assessed by analyses of stable carbon ($\delta^{13}C$) and stable nitrogen ($\delta^{15}N$) isotope ratios (Kelly 2000; Post 2002). As with most laboratory techniques, proper sample preservation and preparation techniques prior to stable-isotope analysis is necessary to maximize analytical precision and to allow for accurate ecological interpretation of results (Gannes et al. 1997; Hobson et al. 1997; Kelly 2000). Reduction of $\delta^{13}C$ variability by removing lipids from tissue samples prior to stable-isotope analysis is often conducted for several reasons. Mainly, ^{13}C in lipids is depleted by as much as 12‰ compared with nonfatty tissues (DeNiro and Epstein 1977; McConnaughey and McRoy 1979), which if left unaccounted can greatly bias estimation of foraging lo-

Received 2 June 2006. Accepted 7 November 2006. Published on the NRC Research Press Web site at http://cjz.nrc.ca on 14 February 2007.

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 doi:10.1139/Z06-187

cations and consumer diets in higher trophic organisms based on sources of primary production when comparing samples with variable lipid content. Also, turnover of ^{13}C in more metabolically active lipids occurs at a faster rate than in proteins (Tieszen et al. 1983), thus proper interpretation of time-integrated isotopic signatures can be confounded in proteinaceous tissue with variable lipid content. Consequently, it is clear that lipid extraction can reduce confounding variation in $\delta^{13}C$ values. Variation in lipid concentration may also influence $\delta^{15}N$ values, but to a lesser degree (Kurle and Worthy 2002; Sotiropoulos et al. 2004).

Nevertheless, the frequency of lipid extraction prior to stable-isotope analysis varies across studies, taxa, and tissue types. Widely accepted criteria for determining the necessity of lipid extraction do not exist and problems arise when results from studies using different sample preparation techniques are compared. Kelly (2000) extensively reviewed the stable-isotope literature on avian and mammalian species and found that lipid extraction was performed in the majority of seabird isotope studies, particularly in muscle and liver samples, but was less frequent in samples from marine mammals and terrestrial herbivores. Lipids have been extracted less frequently from fish muscle samples (Vander Zanden et al. 1999; Jones and Waldron 2003; Cecilio et al. 2004; Ojwang et al. 2004) compared with fattier whole fish samples (Pinnegar and Polunin 1999; Sotiropoulos et al. 2004). Studies examining relations between environmental contaminants and stable isotopes using avian egg components (Jarman et al. 1996; Hobson et al. 2000; Braune et al. 2002) or marine invertebrates (Sato et al. 2002; Fisk et al. 2003) usually extracted lipids prior to analysis.

Kelly (2000) recommended a standardization of lipid extraction in isotope studies. In subsequent studies of lipid extraction effects on isotopes in fish, ^{13}C increased significantly with lipid extraction in fattier whole fish samples but not in leaner dorsal muscle tissue (Pinnegar and Polunin 1999; Sotiropoulos et al. 2004). Two subsequent studies have evaluated lipid effects on isotope ratios in birds, but the direction and magnitude of the effect were not presented in Rocque and Winker (2004), and only differences in $\delta^{13}C$ values between the lipid-free liver tissue and the extracted lipids were tested in Thompson et al. (2000). We know of no peer-reviewed studies that tested effects of lipid extraction on isotope ratios from avian eggs.

Our objective was to determine if lipid extraction and content resulted in statistically and ecologically meaningful shifts in $\delta^{13}C$ and $\delta^{15}N$ values in species from a range of lipid contents and trophic levels. Specifically, we conducted a paired analysis in four different lipid-extracted and non-lipid-extracted sample types (eggs, avian muscle, fish muscle, bivalve tissue) commonly analyzed to help clarify the need for lipid extraction in stable-isotope studies.

Materials and methods

The Aleutian archipelago comprises over 200 volcanic islands that extend westward from the tip of the Alaska peninsula towards Asia, and separates the North Pacific Ocean from the Bering Sea. Ocean-derived nutrients drive high levels of organic production in the coastal ecosystem (National Research Council 1996; Croll et al. 2005), which supports a large population of breeding bald eagles (*Haliaeetus leucocephalus* (L., 1766); Anthony et al. 1999), over 10 million seabirds (Croll et al. 2005), and numerous commercially and ecologically important species of fish (National Research Council 1996). We collected samples from species comprising different trophic levels from the coastal ecosystem of up to seven islands across the entire archipelago, and from several of the Shumagin Islands off the southwestern Alaska peninsula during April–August 1999 through 2002. Samples collected for this study were part of a larger study examining environmental contaminants in the biota of the archipelago, thus specimen collection and subsequent sample compositing matched the larger study design.

We collected 109 fresh eggs from bald eagle nests (1 per nest) located on Kiska, Amchitka, Tanaga, Adak, Amlia, Unalaska, and the Shumagin islands. Eggs were placed in clean, 500 mL plastic containers padded with nest material, and refrigerated until processed. Egg contents were extracted into chemically cleaned jars (VWR brand Trace Clean Quality Assured®) and frozen until analysis. We aged embryos into three broad development categories: early (blood vessel or defined embryo apparent), mid-late (appendages or feathers developed), and nonfertile, because lipid content generally decreases with embryonic development (Peakall and Gilman 1979). Stable-isotope analyses were conducted on aliquots of whole egg samples that had been homogenized previously for analyses of organochlorine contaminants. This precluded subsequent stable-isotope analyses of egg components such as yolk and albumin (e.g., Hobson et al. 2000).

We collected samples from 59 glaucous-winged gulls (*Larus glaucescens* Naumann, 1840), 61 northern fulmars (*Fulmarus glacialis* (L., 1761)), and 58 tufted puffins (*Fratercula cirrhata* (Pallas, 1769)) from Buldir, Kiska, Amchitka, and Adak islands, and 41 rock ptarmigan (*Lagopus muta* (Montin, 1781)) from Kiska, Amchitka, and Adak islands. Tissues from each species were composited into 3–5 samples per island based on collection area. We also collected samples from 5 crested auklets (*Aethia cristatella* (Pallas, 1769)), 4 parakeet auklets (*Aethia psittacula* (Pallas, 1769)), 5 short-tailed shearwaters (*Puffinus tenuirostris* (Temminck, 1835)), 4 black-legged kittiwakes (*Rissa tridactyla* (L., 1758)), 4 common murres (*Uria aalge* (Pontoppidan, 1763)), 4 pelagic cormorants (*Phalacrocorax pelagicus* Pallas, 1811), 4 pigeon guillemots (*Cepphus columba* Pallas, 1811), and 4 Leach's storm petrels (*Oceanodroma leucorhoa* (Vieillot, 1818)) from Buldir Island. Samples from these species were not composited. All seabirds and ptarmigan were collected with shotguns and nontoxic shot and were processed within 1–4 h of collection. An approximate 5 g sample of pectoral muscle was excised from each bird, wrapped in clean aluminum foil, and frozen until analysis.

We collected samples from 57 Pacific halibut (*Hippoglossus stenolepis* Schmidt, 1904), 57 Pacific cod (*Gadus macrocephalus* Tilesius, 1810), 106 rock greenling (*Hexagrammos lagocephalus* (Pallas, 1810)), and 26 rock sole (*Lepidopsetta bilineata* (Ayres, 1855)) from Attu, Buldir, Kiska, Amchitka, and Adak islands. Individuals from each species were composited into 2–5 samples per island based on collection area. Fish were collected using baited long

lines, hook and line, trammel net, or otter trawls and were processed within 1–4 h of collection. An approximate 5–10 g skinless sample of muscle anterior to the dorsal fin was excised from each fish, wrapped in clean aluminum foil, and frozen until analysis.

We analyzed 17 blue mussel samples from sites on Attu, Buldir, Kiska, Amchitka, and Adak islands. Twenty-thirty mussels of representative size classes (15–70 mm) were collected at each site, shelled, the soft tissue composited in chemically clean jars, and frozen. Methods used for all sample collections were approved by the US Geological Survey Animal Care and Use Committee, and authorized by state and federal collection permits.

For lipid-extraction and stable-isotope analyses, all samples were dried at 60 °C for 24–48 h in a drying oven, ground with mortar and pestle into a fine powder, and stored in air-tight 1.7 mL plastic microcentrifuge tubes. We then separated a 0.5–1.5 g subsample for lipid extraction (LE) and another for the non-lipid-extracted (NLE) control. Lipids were extracted using diethyl ether in a Soxhlet apparatus for 48 h (Dobush et al. 1985), then left at room temperature under a fume hood for 8–12 h to let any remaining solvent evaporate. The lipid extracted sample was freeze-dried for 12 h and weighed. Percent lipid was calculated on a dry mass basis. Aliquots (1.0–1.5 mg) of LE and NLE samples were sealed into 5 mm × 9 mm tin capsules (Costech Analytical Technologies Inc., Valencia, California) and analyzed with a Europa Hydra 20/20 continuous flow isotope ratio mass spectrometer at the Stable Isotope Facility, University of California, Davis. We expressed stable-isotope ratios in delta notation as $\delta X = (R_{sample}/R_{standard} - 1) \times 1000$, where δX is the isotope ratio of the sample relative to the standard, and R_{sample} and $R_{standard}$ are the fractions of the heavy to light isotopes in the sample and standard, respectively (i.e., $^{13}C/^{12}C$ and $^{15}N/^{14}N$) (Kelly 2000). Values of δX are expressed in parts per thousands (‰). Measurement error determined from replicate analysis of internal laboratory standards was 0.04‰ for $\delta^{13}C$ and 0.13‰ for $\delta^{15}N$.

Differences in isotope ratios between paired LE and NLE samples were used as the response variable for all statistical hypothesis tests. Except to test overall relations between lipid content and changes in stable-isotope values, we did not combine sample types when testing for differences in $\delta^{13}C$ and $\delta^{15}N$ values to avoid introducing confounding variation owing to different isotopic fractionation among different tissues (Hobson and Clark 1992). We used ANOVA to test for differences in lipid content (%) among sample types and among avian or fish species. We used paired *t* tests to test if lipid extraction changed isotope ratios from whole egg and blue mussel samples. We used ANOVA to test if lipid extraction changed isotope ratios from fish or avian muscle, and categorized species with effects coding to test if the grand mean difference between LE and NLE samples (B_0) differed significantly from 0 while accounting for species effects (Glantz and Slinker 1990). Tukey–Kramer multiple comparison tests were then used to test for differences among species if the grand mean difference was significant. We used simple linear regression for each sample type to determine whether differences in $\delta^{13}C$ and $\delta^{15}N$ values covaried with percent lipid. We conducted separate linear regressions for avian and fish species with $n \geq 12$ to evaluate within-species patterns. Statistical analyses were performed using SAS® version 8.0 (SAS Institute Inc. 2000) and NCSS version 2000 (Hintze 2000).

Results

Percent lipid did not vary significantly ($F_{[2,106]} = 2.7$, $P = 0.07$) with embryo development in whole eggs ($mean_{early} = 29.6\%$, $mean_{mid\text{-}late} = 29.6\%$, $mean_{nonfertile} = 33.4\%$), so all eggs were combined for subsequent statistical analyses. Percent lipid was significantly higher ($F_{[3,288]} = 653.0$, $P < 0.0001$) in whole eggs than in avian muscle, followed by fish muscle and blue mussel tissue (Table 1). Percent lipid differed significantly among avian species ($F_{[11,73]} = 22.3$, $P < 0.0001$), and was highest in black-legged kittiwakes, Leach's storm petrels, glaucous-winged gulls, and northern fulmars and was lowest in rock ptarmigans and pigeon guillemot. Percent lipid did not differ significantly among the fish species ($F_{[3,77]} = 1.2$, $P = 0.31$) (Table 1).

The effects of lipid extraction on $\delta^{13}C$ values followed a pattern similar to differences in percent lipid among sample types. Lipid extraction increased $\delta^{13}C$ values by an average of 2.0‰ from whole eggs ($t_{[108]} = 19.8$, $P < 0.0001$) and 0.6‰ from blue mussels ($t_{[16]} = 4.3$, $P < 0.0001$) (Table 1). ^{13}C values significantly increased in LE avian muscle (mean = 0.8‰) when accounting for variation among species ($t_{[1]} = 14.7$, $P < 0.0001$). Increases in $\delta^{13}C$ values varied among avian species ($F_{[11,72]} = 10.3$, $P < 0.0001$) and was greatest in black-legged kittiwakes, Leach's storm petrels, and glaucous-winged gulls compared with tufted puffins and rock ptarmigan. Lipid extraction also significantly increased $\delta^{13}C$ values in fish muscle when accounting for variation among species ($t_{[1]} = 4.3$, $P < 0.0001$), although the average increase was only 0.2‰. Increases in $\delta^{13}C$ values owing to lipid extraction varied among fish species ($F_{[3,76]} = 4.8$, $P = 0.004$) and was greatest in Pacific halibut and lowest in Pacific cod and rock greenling (Table 1).

Lipid extraction had no significant effect on $\delta^{15}N$ values from whole eggs ($t_{[108]} = -0.3$, $P = 0.62$) or blue mussels ($t_{[16]} = 1.1$, $P = 0.15$) (Table 1). After accounting for species effects, the average difference between $\delta^{15}N$ values from LE and NLE avian muscle did not differ significantly from 0 ($t_{[1]} = -1.9$, $P = 0.07$), but the size and direction of the shift varied among species ($F_{[11,72]} = 2.1$, $P = 0.03$). Lipid extraction slightly increased $\delta^{15}N$ values in muscle tissue from northern fulmars, rock ptarmigan, and crested auklets and decreased for black-legged kittiwakes, common murres, short-tailed shearwaters, and Leach's storm petrels, but the confidence intervals for all species' means overlapped according to the multiple comparison test. Lipid extraction had no effect on $\delta^{15}N$ values among fish species ($t_{[1]} = 1.4$, $P = 0.16$) (Table 1).

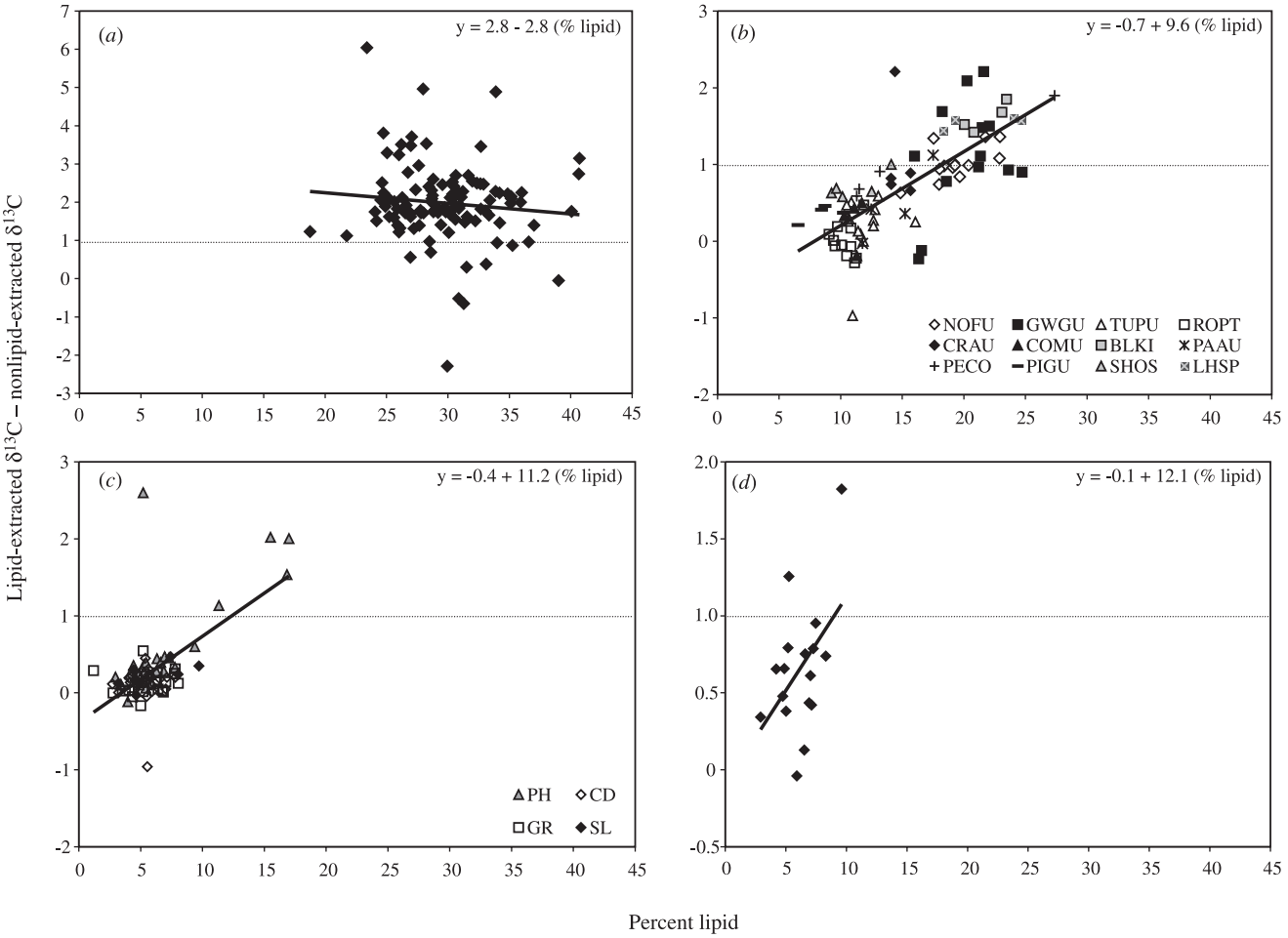
A positive relationship occurred between increasing $\delta^{13}C$ values owing to lipid extraction and lipid content with all sample types combined ($y = -0.1 + 6.8$(% lipid), $t_{[1]} = 16.3$, $P < 0.001$, $R^2 = 0.48$). Within sample types, however, lipid content did not covary with changes in $\delta^{13}C$ values owing to lipid extraction in egg samples ($t_{[1]} = -1.1$, $P = 0.27$, $R^2 = 0.01$; Fig. 1*a*), but the difference between $\delta^{13}C$ values from LE and NLE avian muscle increased significantly with increasing lipid content across all species ($t_{[1]} = 10.3$, $P <$

Table 1. Mean ± 95% CI values for percent lipid (dry mass) and for carbon and nitrogen stable isotopes from lipid-extracted (LE) and non-lipid-extracted (NLE) samples from the Aleutian archipelago, 1999–2002.

				$\delta^{13}C$			$\delta^{15}N$		
Sample type	Species	*n*	Percent lipid	LE	NLE	LE – NLE	LE	NLE	LE – NLE
Whole egg	Bald eagle	109	29.8±0.2A	–17.9±0.2	–19.9±1.2	2.0	14.2±0.2	14.2±0.2	0.0
Avian muscle	Black-legged kittiwake	4	22.2±0.1a	–20.3±0.1	–21.8±0.2	1.5a	11.4±0.3	11.7±0.1	–0.3
	Leach's storm petrel	4	20.6±0.1a	–20.8±0.1	–22.3±0.2	1.5a	11.7±0.6	12.0±0.5	–0.3
	Glaucous-winged gull	13	20.2±0.6a	–18.6±0.6	–19.7±0.9	1.1ab	11.8±0.3	11.9±0.3	–0.1
	Northern fulmar	13	19.4±0.1a	–20.1±0.1	–21.1±0.1	1.0abc	13.2±0.3	13.0±0.2	0.2
	Pelagic cormorant	4	15.9±1.3ab	–18.6±1.3	–19.7±1.6	1.1abc	11.9±0.3	11.7±0.3	0.2
	Crested auklet	5	14.7±0.5b	–22.5±0.5	–23.4±0.1	0.9abc	9.6±1.0	9.1±0.3	0.5
	Short-tailed shearwater	5	11.7±0.4bc	–21.0±0.4	–21.7±0.3	0.7abcd	9.5±0.4	9.8±0.3	–0.3
	Parakeet auklet	4	14.3±0.5bc	–21.6±0.5	–22.1±0.5	0.5bcd	9.9±0.1	9.9±0.2	0.0
	Common murre	4	10.4±0.1bc	–20.6±0.1	–20.8±0.1	0.2cd	10.5±0.6	10.8±0.2	–0.3
	Tufted puffin	13	12.0±0.4bc	–20.9±0.4	–21.1±0.5	0.2d	10.9±0.2	10.8±0.3	1.0
	Pigeon guillemot	4	8.6±0.4c	–17.9±0.4	–18.2±0.3	0.3cd	11.0±0.6	11.3±0.5	–0.3
	Rock ptarmigan	12	10.7±0.2c	–24.4±0.2	–24.4±0.2	0.0d	0.0±1.1	–0.6±1.0	–0.6
	All avian species	85	15.2±2.4B	–20.8±0.4	–21.4±0.4	0.8	9.8±4.3	9.8±4.3	0.0
Fish muscle	Pacific halibut	32	6.6±0.4	–17.3±0.4	–17.8±0.5	0.5a	12.8±0.3	12.8±0.3	0.0
	Rock sole	8	6.2±0.3	–16.4±0.3	–16.6±0.4	0.2ab	11.5±0.3	11.5±0.3	0.0
	Pacific cod	21	5.4±0.4	–17.9±0.4	–18.0±0.4	0.1b	14.0±0.3	13.8±0.3	0.2
	Rock greenling	19	5.5±0.4	–17.0±0.4	–17.1±0.4	0.1b	12.1±0.2	12.0±0.2	0.1
	All fish species	80	5.9±2.4C	–17.3±0.2	–17.5±0.3	0.2	12.8±1.2	12.7±1.1	0.1
Whole body	Blue mussel	17	6.2±0.8C	–18.5±0.4	–19.1±0.4	0.6	5.5±0.4	5.4±0.4	0.1

Note: Values sharing the same letter or values with no letter do not differ (Tukey–Kramer multiple comparison test). Uppercased letters indicate comparisons among sample type for percent lipid, whereas lowercased letters indicate comparisons among species for percent lipid and LE – NLE stable-isotope values.

Fig. 1. Relationships for the difference between lipid-extracted (LE) and non-lipid-extracted (NLE) $\delta^{13}C$ isotope ratios and percent lipid for bald eagle whole eggs (*a*), avian muscle (*b*), fish muscle (*c*), and blue mussels (*d*). A line marking a LE – NLE $\delta^{13}C$ difference of 1‰ is shown on each figure; differences greater than 1‰ exceed the range of expected $\delta^{13}C$ variability owing to trophic enrichment. Avian species and corresponding codes are glaucous-winged gull (*Larus glaucescens*; GWGU), northern fulmar (*Fulmarus glacialis*; NOFU), tufted puffin (*Fratercula cirrhata*; TUPU), rock ptarmigan (*Lagopus muta*; ROPT), crested auklet (*Aethia cristatella*; CRAU), common murre (*Uria aalge*; COMU), black-legged kittiwake (*Rissa tridactyla*; BLKI), parakeet auklet (*Aethia psittacula*; PAAU), pigeon guillemot (*Cepphus columba*; PIGU), pelagic cormorant (*Phalacrocorax pelagicus*; PECO), short-tailed shearwater (*Puffinus tenuirostris*; SHOS), and Leach's storm petrel (*Oceanodroma leucorhoa*; LHSP). Fish species and corresponding codes are Pacific halibut (*Hippoglossus stenolepis*; PH), Pacific cod (*Gadus macrocephalus*; CD), rock greenling (*Hexagrammos lagocephalus*; GR), and rock sole (*Lepidopsetta bilineata*; SL).



0.001, R^2 = 0.56; Fig. 1*b*). The slope of the avian muscle regression crossed a 1‰ change in $\delta^{13}C$ values owing to lipid extraction at approximately 20% lipid. Within those avian species that had adequate sample sizes, lipid content positively covaried with increasing $\delta^{13}C$ values owing to lipid extraction in northern fulmars ($t_{[1]}$ = 2.7, P = 0.02, R^2 = 0.35), a species with relatively high lipid content. However, the relationship between changes in $\delta^{13}C$ values and lipid content was not significant for glaucous-winged gulls (which had lipid content similar to northern fulmars) or leaner tufted puffins and rock ptarmigan ($t_{[1]} \leq 1.5$, $P \geq 0.15$, $R^2 \leq 0.10$). Increasing lipid content also covaried with lower $\delta^{13}C$ values in NLE fish muscle samples across all species ($t_{[1]}$ = 7.2, $P < 0.001$, R^2 = 0.39), and the slope of the regression crossed a 1‰ change in $\delta^{13}C$ values owing to lipid extraction at approximately 12% lipid (Fig. 1*c*). Within species, however, only lipid content from Pacific halibut positively covaried with increasing $\delta^{13}C$ values owing to lipid extraction ($t_{[1]}$ = 5.7, $P < 0.0001$, R^2 = 0.51). Changes in $\delta^{13}C$ values were not associated with lipid content for any other fish species ($t_{[1]} \leq 2.0$, $P \geq 0.09$, $R^2 \leq 0.31$). Lipid content was not related strongly to changes in $\delta^{13}C$ values in blue mussels ($t_{[1]}$ = 2.0, P = 0.06, R^2 = 0.16) (Fig. 1*d*).

Lipid content was unrelated to changes in $\delta^{15}N$ values across all sample types ($t_{[1]}$ = –0.7, P = 0.51, R^2 = 0.00). Similarly, lipid content did not covary with changes in $\delta^{15}N$ values within whole eggs ($t_{[1]}$ = 1.0, P = 0.33, R^2 = 0.00), avian muscle ($t_{[1]}$ = –0.18, P = 0.85, R^2 = 0.00), or fish muscle ($t_{[1]}$ = –1.1, P = 0.27, R^2 = 0.00) samples. Relationships between changes in $\delta^{15}N$ values and lipid content were not significant within any avian ($t_{[1]} \leq 2.0$, $P \geq 0.07$ $R^2 \leq$

0.23) or fish ($|t|_{[1]} \leq 1.5$, $P \geq 0.18$, $R^2 \leq 0.15$) species. In contrast, lipid content positively covaried with increasing $\delta^{15}N$ values from LE blue mussel samples ($t_{[1]} = 2.9$, $P = 0.01$, $R^2 = 0.32$).

Discussion

Carbon isotopes

Lipid extraction resulted in significantly higher $\delta^{13}C$ values across all sample types. However, our results should also be viewed in an ecological context by examining the extent to which shifts in isotope values associated with lipid extraction could confound identification of carbon sources. Patterns of $\delta^{13}C$ are often interpreted on relative rather than absolute scales, where ^{13}C becomes more depleted as the proportion of pelagic production incorporated into the food web increases (e.g., Hobson et al. 1994; Jarman et al. 1996; Ojwang et al. 2004). Sources of primary production in marine environments can be partitioned because benthic algae are exposed to thicker diffusive boundary layer conditions compared with pelagic phytoplankton occupying more turbulent waters, which ultimately causes greater ^{13}C enrichment in benthic algae compared with phytoplankton (France 1995). As a result, ^{13}C in consumers deriving carbon from benthic or nearshore algae is typically enriched by 5‰ compared with consumers deriving carbon from pelagic phytoplankton (France 1995). Conversely, relatively little ^{13}C enrichment (<1‰) occurs with each trophic step (Fry and Sherr 1984; Kelly 2000), which allows $\delta^{13}C$ values to be a reliable signature for primary production across trophic levels.

Lipid extraction significantly increased $\delta^{13}C$ values in bald eagle whole eggs, which were the fattiest sample type analyzed in our study. Bald eagles in the Aleutian archipelago have wide diet breadths and often forage on prey from offshore habitats (e.g., northern fulmars, marine mammal carcasses), which come into proximity of the eagles' foraging range on the coastline in addition to prey from nearshore environments (e.g., sea otter pups, kelp forest fishes) (Anthony et al. 1999). The average decrease of 2.0‰ in NLE whole eggs may be large enough to bias interpretation of pelagic vs. nearshore carbon signatures, thus variation owing to lipid content should be removed. Interestingly, there was no consistent pattern of changing $\delta^{13}C$ values across the wide range of lipid contents (18%–42% dry mass) in bald eagle eggs, which suggests a simple correction factor of 2.0‰ (the average difference between LE and NLE) values could be added to $\delta^{13}C$ values from NLE whole bald eagle egg samples. This correction should only be applied to whole homogenized eggs because ^{13}C can fractionate differently by egg component (Hobson et al. 2000).

Lipid extraction also significantly increased $\delta^{13}C$ values from avian muscle, but average differences in $\delta^{13}C$ values between LE and NLE muscle samples did not exceed 1.5‰ for any species. Kelly (2000) also found a similar difference of 1.3‰ between LE and NLE $\delta^{13}C$ values in the seabird stable-isotope literature. The ecological significance of lower $\delta^{13}C$ values in NLE samples from avian muscle in our study was apparently species dependent and more sensitive to variable lipid content. Differences in $\delta^{13}C$ values of ≤1‰ for most of our avian species were within the range of expected variation associated with trophic enrichment (1‰) and should not confound relative interpretation of carbon sources. However, lower $\delta^{13}C$ values in NLE samples were highly correlated with increasing lipid content across all avian species, so the likelihood of confounding pelagic vs. benthic ^{13}C signatures, and subsequent need for lipid extraction, generally increases for more lipid-rich species. Causes for this pattern are complex but may be related to variable diets among species and differential incorporation of dietary lipids into consumer tissues (Thompson et al. 2000; Sotiropoulos et al. 2004). For example, low lipid content and lack of change in $\delta^{13}C$ values in rock ptarmigan muscle in our study probably reflected a largely herbivorous diet devoid of lipid-rich vegetation (Holder and Montgomerie 1993) and the incorporation of relatively few dietary lipids. Conversely, the higher muscle lipids and lower $\delta^{13}C$ values in NLE samples from piscivorous species such as black-legged kittiwakes and pelagic cormorants are probably related to consumption of a more lipid-rich fish diet (Baird 1994; Hobson 1997), or in the case of procellariformes (e.g., northern fulmars, Leach's storm petrels) is likely reflected by their physiological ability to concentrate dietary lipids into stomach oils and incorporate lipid-carbon into protein (Thompson et al. 2000). The within-species correlation between lipid content and lower $\delta^{13}C$ values owing to lipid extraction in northern fulmars may further reflect their wide diet breadth with variable dietary lipids ranging from zooplankton to blubber from dead marine mammals (Hatch and Nettleship 1998). Glaucous-winged gulls, a larid, had similar lipid contents and changes $\delta^{13}C$ values owing to lipid extraction compared with northern fulmars. However, the lack of a significant within-species correlation between changing $\delta^{13}C$ values and lipid content among glaucous-winged gulls may suggest either a lack of biochemical pathways that efficiently incorporate ^{13}C depleted lipid-carbon into protein as in procellariformes, or diets of some individual gulls in the Aleutians have high proportions of intertidal invertebrates (e.g., sea urchins, chitins, limpets) (Irons et al. 1986) compared with more lipid-rich items.

Changes in $\delta^{13}C$ values owing to lipid extraction were substantially less in fish muscle than in all other sample types, and the average difference between LE and NLE of 0.2‰ probably would not confound relative interpretation of carbon source signatures. These results concur with Sotiropoulos et al. (2004), who found marginal and ecologically insignificant ^{13}C depletion in NLE muscle compared with fattier whole fish samples and who ostensibly provided justification for the lack of lipid extraction in isotope studies that used fish muscle. Decreases in $\delta^{13}C$ values from NLE samples were explained by lipid content in fish muscle across all species, which was similar to the patterns observed in muscle samples across all avian species in our study and among rainbow trout (*Oncorhynchus mykis* (Walbaum, 1792)) tissue types in the study by Pinnegar and Polunin (1999). Variation in lipid content may be partly explained by differences in dietary lipids of the fish species sampled in our study (Kling et al. 1992; Sotiropoulos et al. 2004). For example, Pacific halibut have wide diet breadths and often shift from invertebrates to fish as they increase in age and size (International Pacific Halibut Commission 1998). Several halibut samples (31%) came from fish over

20 kg, and while our compositing scheme precluded correlating size and lipid content, possible differences in dietary lipids related to size may explain why Pacific halibut had the greatest difference between LE and NLE $\delta^{13}C$ values, which also was significantly correlated with lipid content.

Evaluation of lipid effects on stable isotopes in blue mussels has particular importance for food-web studies. Isotopic composition of freshwater mussels reflects the spatial and temporal isotopic baseline in freshwater systems necessary for accurately estimating trophic position of consumers in freshwater food webs (Post 2002). Presumably, stable isotopes from blue mussels should provide useful baseline isotopic signatures in nearshore marine environments as well. Like all other sample types, $\delta^{13}C$ values were significantly lower in NLE blue mussel samples, but the mean difference between LE and NLE of 0.6‰ should not greatly confound relative carbon source tracking and bias isotopic baselines. However, blue mussels can feed on both phytoplankton and zooplankton (Wong and Levinton 2004), so the effects of lipid content on carbon isotopes from grazing herbivores such as limpets or littorines requires further study.

Nitrogen isotopes

Nitrogen isotope values did not vary significantly owing to lipid extraction for any species or sample type. More importantly, average differences between LE and NLE samples did not exceed ±0.6‰, which is far less than the typical 3‰–4‰ enrichment observed between trophic levels in high-latitude environments (Hobson and Welch 1992; Hobson et al. 1994; Fisk et al. 2003). Moreover, lipid extraction did not consistently increase or decrease $\delta^{15}N$ values, particularly among avian muscle, and lipid content did not explain significant variation in $\delta^{15}N$ values among most sample types and species in our study. The possible exception was the positive covariation between lipid content and increasing $\delta^{15}N$ values from LE blue mussel samples that indicated lipid content may inflate $\delta^{15}N$ baseline calculations. However, the mean difference between LE and NLE across the range of lipid content in blue mussels was only 0.1‰, so it is unlikely that lipid content has a strong biological effect on $\delta^{15}N$ in relatively lean blue mussel tissues.

Trophic level calculations are also highly sensitive to variable fractionation of $\delta^{15}N$ compared with that of $\delta^{13}C$ (Post 2002). Therefore, potential variation in $\delta^{15}N$ owing to lipid content requires assessment. In our study, average changes in $\delta^{15}N$ values owing to lipid extraction ranged from –0.6‰ to 1.0‰. Post (2002) conducted an extensive literature survey and calculated an average $\delta^{15}N$ trophic fractionation of 3.4‰, with a standard deviation of 1.0‰. Changes in $\delta^{15}N$ values owing to lipid extraction in our study fell within the range of natural variation for $\delta^{15}N$ trophic fractionation, so the use of NLE $\delta^{15}N$ samples should not bias trophic-level calculations.

Other studies found some changes in $\delta^{15}N$ values associated with lipid extraction in a variety of sample types. For example, Kurle and Worthy (2002) found lower $\delta^{15}N$ values in NLE blubber from northern fur seals (*Callorhinus ursinus* (L., 1758)), but suggested effects of lipid extraction might be diminished in less lipid-rich tissue, assuming that ^{15}N enrichment is a function of the ratio of protein to lipid per unit tissue. Sotiropoulos et al. (2004) detected slightly higher ^{15}N depletion (0.3‰–0.5‰) in NLE fish muscle. However, they used chloroform–methanol, which is not a lipid-specific solvent and can bind to and remove substantial amounts of protein (Dobush et al. 1985). It did not appear that diethyl ether recommended by Dobush et al. (1985) affected $\delta^{15}N$ values in our study. Thus, removing lipids with chloroform–methanol may cause inflated $\delta^{15}N$ values, and the type of solvent used to extract lipids prior to stable-isotope analysis should be considered carefully.

Conclusions

The necessity of lipid extraction in stable-isotope studies depends on study objectives and types of sample and species collected. Changes >1‰ in $\delta^{13}C$ values associated with lipid extraction and content are above the expected range of variation owing to trophic fractionation and are more likely to become ecologically significant as they approach 5‰ (France 1995). Lean avian and fish muscle sample types in our study with average ^{13}C loss of <1‰ seemed less prone to ecologically significant changes in $\delta^{13}C$ values compared with bald eagle eggs. However, the potential for confounding primary production sources increased with higher tissue lipid content across all sample types. In our study, ^{13}C loss of >1‰ was generally associated with lipid contents >15%–20% (Fig. 1). Lipid extraction is likely not necessary for most seabird and fish muscle tissues when carbon isotopes are interpreted on a relative scale. This has useful implications for streamlining sample preparation of lean muscle tissues widely used in stable-isotope studies partly because of their intermediate turnover rate (Tieszen et al. 1983; Hobson and Clark 1992). In contrast, lipid extraction should be performed on fatty sample types such as whole egg homogenates and lipid-rich avian species, or when comparing values across previous isotope studies that used lipid-extracted tissues. Lipid extraction is probably not necessary for the sample types analyzed in this study if $\delta^{15}N$ is the only isotope ratio of interest. Caution should be exercised, however, when extrapolating our results across an entire food web, since our study focused primarily on higher trophic level organisms with relatively low lipid contents.

There is an important situation not tested in this study that requires consideration when evaluating the need for lipid extraction. Mixing models based on mass balance equations use stable-isotope values to determine the contribution of different prey items to a consumer's diet. These models are used increasingly in studies of diet composition and overlap, but are often complex and sensitive to slight isotopic variations (Phillips and Gregg 2001). Pinnegar and Polunin (1999) illustrated that estimates of consumer diets from mass balance equations are highly sensitive to small (<1.16‰) variations in $\delta^{13}C$ and $\delta^{15}N$ associated with lipid content, especially if the range of isotope values in consumer tissues is small. Thus, lipid extraction should be performed when maximum estimate precision is needed for calculations of diet composition and overlap, and our results provide a useful construct for future research to further evaluate the effects of lipid extraction on mixing model results.

Lastly, expenses associated with lipid extraction more than doubled our analytical costs, which is an unfortunate but necessary consideration for researchers with limited budgets and laboratory use.

Acknowledgements

This project was funded by the US Navy through contract No. N68711-02-MP-C2003 to the Western Ecological Research Center, US Geological Survey, administered by J. Estes. The US Fish and Wildlife Service, Alaska Maritime National Wildlife Refuge provided logistical and scientific support; in particular, G.V. Byrd, J. Williams, J. Mueller, and the crew of the M/V *Tiglax*. We thank T. Tinker, W. Jarman, and numerous personnel for sample collection and processing. We thank J. Yee and F. Weckerly for statistical advice and J. Estes for comments on previous drafts of the manuscript. The Aleut, Ounalashka, and Shumagin corporations allowed access to their lands for bald eagle work.

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